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GROWTH OF SOME PARASITIC FUNGI IN SYNTHETIC CULTURE MEDIA

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In some studies on parasitism being conducted by the writers, it early became imperative that considerable attention be given to synthetic nutrient solutions for fungi. Evidence points strongly to food as being at least one of the dominant factors in the type of growth produced, and in the variations obtained. The presence or absence of any of the essential inorganic elements may vary the growth forms to such an extent that structural changes may develop. Striking responses to chemicals have been noted, in color changes as shown by Milburn (8) and Bessey (1), by generic changes as in the presence or absence of setae in *Colletotrichum lindemuthianum*, and of conidial chains in *Alternaria brassicae* and *Macrosporium brassicae*, as reported by Stevens (15), and by changes in the hydrogen-ion concentration during growth of the organism. The last is undoubtedly a factor in parasitism.

The work of Pasteur (10) on yeasts led to a very extensive investigation of the food requirements of fungi. The early investigators dealt in a minor way with the inorganic foods, more emphasis being given to the organic compounds which could be used as a source of carbon. It was early recognized, however, that for a detailed study of any fungus the food supply had to be fairly definitely controlled and that synthetic solutions rather than plant decoctions should be used.

Of the commonly used synthetic solutions, that developed by Raulin (12)[2] is the earliest. The solution contains most of the elements found in the ash of fungi. For the most part it was developed to meet the requirements for the growth of *Aspergillus niger* and of some other strict saprophytes.

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[1] Contribution from the Department of Botany, Michigan Agricultural College.

[2] Raulin's synthetic solution:

Ammonium nitrate	4.0 g.
Ammonium phosphate	0.6 g.
Ammonium sulphate	0.25 g.
Potassium silicate	0.07 g.
Potassium carbonate	0.4 g.
Zinc sulphate	0.07 g.
Ferric sulphate	0.07 g.
Magnesium carbonate	0.4 g.
Saccharose	70.00 g.
Tartaric acid	4.00 g.
Water	1500.00 cc.

Mayer (7)[3] prepared a similar solution, eliminating zinc, iron, and silicon, but including calcium acid phosphate in addition to the other elements.

Simpler synthetic solutions were later devised by Pfeffer (11),[4] Richards (13),[5] Currie (3),[6] Uschinsky (5),[7] and Czapek (4),[8] and these are frequently used in present-day studies.

[3] Mayer's synthetic solution:

Magnesium sulphate	2.5 g.
Ammonium nitrate	10.0 g.
Calcium phosphate, $Ca_3(PO_4)_2$	2.5 g.
Potassium acid phosphate (monobasic)	5.0 g.
Cane sugar	50.0 g.
Water	1000.0 cc.

[4] Pfeffer's solution:

Ammonium nitrate	10.0 g.
Potassium phosphate (monobasic)	5.0 g.
Magnesium sulphate	2.5 g.
Cane sugar	50.0 g.
Ferrous sulphate	trace
Water	1000. cc.

Reaction, pH = 4.3

[5] Richards' solution:

Potassium nitrate	1. g.
Potassium acid phosphate (monobasic)	0.5 g.
Magnesium sulphate	0.25 g.
Ferric chloride	trace
Saccharose	3.43 g.
Water	100. cc.

Reaction, pH = 4.2

[6] Currie's solution:

Ammonium nitrate	2. g.	2.5 g.
Potassium acid phosphate (monobasic)	.75 g.	1.0 g.
Magnesium sulphate	.25 g.	.25 g.
Cane sugar	125.00 g.	150.00 g.
Water	1000 cc.	

Reaction (add 4 cc. of $N/5$ HCl), pH = 3.4–3.6

[7] Uschinsky's solution:

Ammonium lactate	6.7 g.
Sodium asparaginate	3–4. g.
Potassium acid phosphate (monobasic)	2–2.5 g.
Magnesium sulphate	0.3–0.4 g.
Sodium chloride	5–7. g.
Calcium chloride	0.1 g.
Glycerin	30–40. g.
Water	1000. cc.

[8] Czapek's solution:

Magnesium sulphate	0.5 g.
Potassium acid phosphate (monobasic)	1.0 g.
Potassium chloride	0.5 g.
Sodium nitrate	2.0 g.
Cane sugar	3.4 g.
Water	100. cc.

Reaction, pH = 6.8

Coons (2),[9] working with *Plenodomus fuscomaculans*, recognized that a medium favorable to vegetative growth is not necessarily conducive to sporulation. He found it necessary to develop two solutions, one inducing mycelial development and the other favoring spore production.

In a general way all the above mentioned solutions are similar. Emphasis is most frequently placed on the source of carbon. In no case is there given a method for the arranging of the proportions and concentrations. The presence or absence of any one of the inorganic constituents depends upon whether the investigator considered it important for the growth of the particular fungus under experimentation. Most discussion, however, has centered around the rôles of calcium, iron, and zinc.

Molisch (9) is of the opinion that calcium is not necessary for the normal development of fungi. While this fact is based on little experimental data, it has been generally accepted.

Currie (3) has concluded that the exclusion of iron is without effect on the growth of *Aspergillus niger*. Other investigators have found that, although iron and calcium may not be elements essential for growth, they unquestionably exert a beneficial effect on the growth of many fungi. More recently the work of Steinberg (14) has shown that in the case of *Aspergillus niger*, zinc has a very decided stimulative effect on the quantity of fungus produced. He states that one reason that zinc has not previously been used in such media is that sufficient amounts were dissolved from the glassware to supply this need. This same statement might well be made in the case of iron and possibly in that of some of the other essential inorganic elements.

It is worthy of note that nearly all the synthetic solutions now in general use have been formulated on the basis of the food requirements of strict saprophytes, in most cases with *Aspergillus niger*, a fungus that is not at all selective but will grow on an exceptionally wide range of media. Moreover, the name *Aspergillus niger* as used by different investigators represents rather a group of related fungi than one species. For these reasons, a study of the rôle of any salt cannot be safely made when such types of fungi are used as criteria.

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[9] Coons' cheap synthetic solution:

Magnesium sulphate	1.23 g.
Potassium acid phosphate (monobasic)	2.72 g.
Potassium nitrate	2.02 g.
Maltose	7.2 g.
Water	1000. cc.

Coons' synthetic solution:

Magnesium sulphate	1 cc. of $M/5$ solution
Potassium acid phosphate	5 cc. of $M/5$ solution
Asparagin	1 cc. of $M/5$ solution
Maltose	5 cc. of $M/5$ solution
Water	50 cc.

The growing of strictly parasitic fungi in synthetic culture solutions has not come into general practice. On the contrary, various circumstances have led to the general use of plant decoctions. Such media are excellent for certain purposes, but for physiological work media of known composition are almost indispensable.

In an attempt to formulate a synthetic solution for the growth of parasitic fungi, the rôles of the so-called essential inorganic elements along with that of calcium and zinc were studied. For this work Richards' solution was used as a standard and for the deviations. The inorganic constituents were replaced by non-essentials, the concentrations and balance being maintained as nearly as possible. In addition, eight sugars were used. The possible importance of the presence of minute quantities of zinc and of other substances was recognized, and special precautions were taken in selecting glass of tested solubility and in its thorough cleaning. Erlenmeyer flasks of 150-cc. capacity were employed. The water used was stock distilled water redistilled through a Branstead still. The chemicals were obtained from the J. T. Baker Chemical Company and from the lot prepared for the use of the National Research Council in nutrition studies of higher plants. The arrangement of the experiment is given in table 1. In each flask was placed 50 cc. of the nutrient solution, and each solution was set up in three sets of triplicates. The solutions were sterilized by heating in an autoclave 30 minutes at 10 pounds' pressure. One series of flasks was inoculated with *Fusarium oxyspórum*, another with *Rhizopus nigricans*, and a third with *Aspergillus niger*. After the flasks were inoculated, they were placed at room temperature and the organisms were allowed to grow for 16 days. At the end of this period the mycelium and spores were collected on previously dried and weighed filter paper, dried to constant weight in a steam oven at 97° C., and reweighed. Results are recorded in table 1.

The results of this experiment indicate that calcium exerts a stimulative action on the growth of the three organisms used. The exact manner in which calcium influences growth is problematical. However, it can be logically concluded that its rôle of counteracting acidity in higher plants plays an important part in the growth of these fungi, especially in that of *Aspergillus niger* and *Rhizopus nigricans*, two acid producers. To make certain that the increased weight was due to fungous growth and not to calcium oxalate crystals, the mycelium was treated with alcohol and thirty percent hydrochloric acid and redried. The treatment resulted in no appreciable decrease in weight of the fungous material grown with calcium present, as compared with the cultures grown without calcium. The quantity of acid produced by the growth of *Aspergillus niger* and *Rhizopus nigricans* is proportional to the amount of fungous material produced. With available carbon, growth continues until stopped by the hydrogen-ion concentration. If the acid is neutralized, *Rhizopus nigricans* will grow until

TABLE I

No. of Solution	Composition of Culture Solution	*Fusarium oxysporum*, Dry Weight in Grams*	pH	*Aspergillus niger*, Dry Weight in Grams*	pH	*Rhizopus nigricans*, Dry Weight in Grams*	pH
1	Richards' solution	.2094	5.8	.5450	2.4	.2015	2.6
2	Richards' solution, KNO_3 replaced by $Ca(NO_3)_2$	.2494	6.4	.8070	4.8	.2747	
3	Richards' solution, KH_2PO_4 replaced by Na_2HPO_4	.2575	6.4	.3841	2.6	.1843	
4	Richards' solution, KH_2PO_4 replaced by KCl	.0736	4.4	.1641		.0925	
5	Richards' solution, KNO_3 replaced by KCl	No growth		.0250	4.6	No growth	
6	Richards' solution, sucrose replaced by dextrose	.1932	5.6	.3700	3.6	.1234	
7	Richards' solution, sucrose replaced by maltose	.3124	6.4	.3560	2.4	.1540	
8	Richards' solution, sucrose replaced by lactose	.0404	5.0	.2640	4.0		
9	Richards' solution, sucrose replaced by glycerin	.1163	4.2	.0406			
10	Richards' solution, sucrose replaced by raffinose			.2900	2.4	.1000	
11	Richards' solution, sucrose replaced by galactose	.2345	6.0	.4240	2.6	.1560	
12	Richards' solution, sucrose replaced by levulose	.1132	4.2	.1690	3.0	.0825	
13	Richards' solution, sucrose replaced by mannose	.2146	5.8	.4040	3.2	.1420	
14	Richards' solution, iron omitted	.2458	6.4	.7243	3.6		
15	Richards' solution, plus 15 mg. $ZnSO_4$	.2432	6.4	.7131			
16	" " " 10 " "	.2243	6.2	.9043	2.4		
17	" " " 5 " "	.2186	6.3	.8078	2.4		
18	" " " 1 " "	.2506	6.6	.8124	2.4		
19	" " " 0.5 " "			.6312	2.8		
20	Coons' cheap synthetic solution	.0500	3.8				

* Average for three cultures.

the available carbon is completely used up. Our results in this connection are contrary to those of Graves (6), who states that a thermolabile "staling" substance is produced which stops the growth of the fungus.

The reaction of the culture solution in which *Fusarium oxysporum* was grown continued to become acid until a hydrogen-ion concentration of pH 3.6 was reached, then turned alkalineward, and growth continued until all the organic compounds were broken up and a hydrogen-ion concentration of pH 8.4 was reached.

Zinc sulphate stimulated growth in the case of *Aspergillus niger* but not in that of *Fusarium oxysporum* or of *Rhizopus nigricans*.

Since calcium proved to be so beneficial when placed in the nutrient solution for the growth of the organisms used in the preceding experiment, it was thought important to determine whether this same relation would hold for a number of parasitic organisms. An experiment was planned using Richards' solution as a medium in one series and the same solution except with calcium nitrate replaced by potassium nitrate in a second series. A third series with zinc sulphate added to Richards' solution was arranged. These series were prepared in triplicate and with the same precautions as used in the preceding experiment. The organisms used were selected to represent as wide a range of parasites as could be obtained from the stock cultures of the laboratory. The experiment was continued for each organism until growth seemed to stop. The organisms used and the results are tabulated in table 2.

TABLE 2

Organism	Richards' Solution, Calcium Nitrate Substituted for Potassium Nitrate		Richards' Solution		Richards' Solution Plus 5 Mg. Zinc Sulphate	
	Dry Wt.*	pH	Dry Wt.*	pH	Dry Wt.*	pH
Botrytis allii	.2199	4.2	.2404	5.8	.2191	6.3
Phoma apiicola	.2798	5.2	.1427	6.0	.1963	6.0
Fusarium conglutinans	.1628	6.0	.1669	6.4	.1560	6.6
Cercospora apii	.3750	5.2	.3003	5.4	.3509	5.4
C. beticola	.3451	6.4	.3411	6.8	.3118	6.8
Fusarium batatatis	.2391	6.1	.2377	6.3	.2501	8.0
F. radicicola	.2666	8.0	.3224	6.8	.2561	7.0
Sclerotinia libertiana (strain 1)	.4545	3.8	.4675	4.2	.4391	3.8
S. cinerea	.2132					
S. libertiana (strain 2)	.1963	3.0	.2153	3.0	.2052	3.0
Rhizoctonia solani	.1055		No growth		No growth	
Macrosporium sarcinaeforme	.2777	6.8	.2801	6.6	.2683	6.6
Sphaeropsis malorum	.3139	3.6	.3751	6.8	.3689	6.8
Sterigmatocystis violae	.3264	6.0	.2884	6.0	.2435	6.0
Vermicularia sp.	.3272		.3563		.3363	
Ascochyta pisi	.2677	6.2	.3782	6.4	.3278	8.0
Colletotricum lagenaria	lost					
Dothidella quercus	.3652	6.6	.2411	6.5	.2999	6.5

* Average for three cultures.

Summarizing the results of table 2, calcium is found to be generally beneficial. In the case of four organisms it retarded growth. In the remaining cultures it was stimulative or exerted no action. *Rhizoctonia solani* grew only when calcium was present. The importance of the rôle of calcium, however, cannot be estimated from the results of this experiment, as we have no proof that we have been using it in its proper proportions.

Zinc sulphate gave slightly beneficial effects with only two organisms, *Dothidella quercus* and *Phoma apiicola*.

A striking example of color formation was shown in the case of *Fusarium radicicola* which was pink in the solution containing calcium and colorless in potassium solutions. The color was destroyed when hydrochloric acid was added and could not be obtained again by the addition of sodium hydroxide.

The results of the above described experiment indicate that calcium might be classed as one of the essential inorganic elements in synthetic solutions for some fungi. Moreover, it would seem that the salt requirements for an optimum synthetic solution would be composed of the six elements, nitrogen, phosphorus, sulphur, potassium, calcium, and magnesium.

We have no evidence to the effect that we have been using these inorganic elements in correct proportions. Since the salt requirements for fungi are probably the same as for higher plants, a series of culture solutions was arranged according to the method used by the National Research Council for work in the study of nutrition in higher plants. A triplicate series of twenty-one cultures, using potassium acid phosphate (monobasic), calcium nitrate, and magnesium sulphate, was arranged. The salts varied in the different solutions by increments of one eighth and had an osmotic concentration of three and one half atmospheres. Sucrose was added in equal amounts of 3.43 grams per 100 cc. of the solution, thereby giving the culture solutions a total osmotic concentration of four and one half atmospheres. Similarly, a triplicate series was arranged, using potassium nitrate, calcium acid phosphate, and magnesium sulphate.

TABLE 3

No.	Cc. of *M*/14 KH_2PO_4	Cc. of *M*/14 $Ca(NO_3)_2$	Cc. of *M*/14 $MgSO_4$	Cc. of Sugar Sol., 3.43 g. in 10 cc.	Water to make 50 cc.	Cc. of *M*/3.5 KNO_3	Cc. of *M*/35 $Ca(H_2PO_4)_2$	Cc. of *M*/3.5 $MgSO_4$	Cc. of Sugar Sol., 3.4 g. in 10 cc.	Water to make 50 cc.
11	4	4	22.4	5	14.6	.95	9.5	5.7	5	28.85
12	3.5	6.8	17.2	5	17.5	.91	18.6	4.6	5	20.89
13	3.4	10	13.2	5	18.4	.84	25.6	3.43	5	15.13
14	3.1	12.4	9.4	5	20.1	.81	32.6	2.45	5	9.14
15	3.1	15	6	5	20.9	.74	37.11	1.47	5	5.68
16	3.0	17	2.8	5	22.2	.73	43.75	.74	5	.00
21	7.4	4	17.2	5	16.4	1.89	9.5	4.62	5	29.03
22	7.0	6.8	13.2	5	18.0	1.68	18.6	3.43	5	21.39
23	6.5	10.0	9.4	5	19.6	1.58	25.6	2.45	5	17.27
24	6.2	12.4	6.0	5	20.4	1.47	32.6	1.47	5	9.46
25	6.0	15.0	2.8	5	21.2	1.44	37.1	.74	5	5.72
31	10.6	4.0	13.2	5	17.2	2.63	9.5	3.43	5	29.44
32	10.0	6.8	9.4	5	18.8	2.45	18.6	2.45	5	21.50
33	9.5	10.0	6.0	5	19.5	2.35	25.6	1.47	5	15.58
34	9.0	12.4	2.8	5	20.8	2.24	32.6	.74	5	9.42
41	13.8	4.0	9.4	5	17.8	3.47	9.5	2.45	5	29.58
42	13.2	6.8	6.0	5	19.0	3.25	18.6	1.47	5	21.68
43	12.6	10.0	2.8	5	19.6	2.98	25.6	.74	5	15.68
51	17.2	4.0	6.0	5	17.8	4.38	9.5	1.74	5	29.65
52	16.4	6.8	2.8	5	19.0	3.96	18.6	.74	5	21.70
61	20.3	4.0	2.8	5	17.9	4.87	9.5	.74	5	29.89

Osmotic concentration, 4.5 atm.; pH, 4.8–5.2.

The method of arranging the concentrations is given in table 3. The inorganic salts used were of the highest purity of the grade mentioned above. The technique used was the same as in the preceding experiments. Special care was taken in inoculation of solutions so as to obtain uniform growths at the outset. *Fusarium oxysporum*, *Macrosporium sarcinaeforme*, and *Phoma apiicola* were used. Richards', Pfeffer's, Currie's, Meyer's, and Czapek's solutions were used as checks.

Dry weights were obtained after the cultures had grown for sixteen days. The hydrogen-ion concentration of each culture solution was determined at the time the experiment was concluded. The results obtained are given in table 4.

TABLE 4

KH_2PO_4, $Ca(NO_3)_2$, $MgSO_4$, KNO_3, $Ca(H_2PO_4)_2$, $MgSO_4$

No. of Culture	*Fusarium oxysporum*			*Fusarium oxysporum*			*Macrosporium sarcinaeforme*			*Phoma apiicola*		
	Dry Wt.*	pH	Rank	Dry Wt.*	pH	Rank	Dry Wt.*	pH	Rank	Dry Wt.*	pH	Rank
11	.2159	5	11	.1514	3.6	15	.4311	6	17	.3448	6.3	7
12	.2163	6.3	10	.1422	3.6	17	.3862	5.7	20	.1627	5.8	12
13	.2221	6.4	9	.1369	3.8	18	.4565	5.4	16	.1514	4.8	14
14	.1876	3.6	17	.1216	4.0	19	.4085	6.2	18	.1567	3.8	13
15	.1765	5.4	19	.1160	4.0	20	.3414	5.0	21	.1019	3.5	19
16	.1895	6.4	16	.0985	4.6	21	.3902	5.0	19	.0269	3.4	21
21	.2029	3.9	13	.2133	5.4	21	.6888	7.0	11	.5301	6.5	3
22	.1755	6.2	20	.2326	5.8	9	.8002	6.0	8	.1265	4.4	16
23	.2246	6.4	7	.2089	5.0	12	.6708	5.2	13	.1764	3.8	11
24	.2601	5.4	2	.1898	5	14	.4904	4.8	15	.0815	3.8	20
25	.2548	4.6	3	.1457	5	15	.6352	5	14	.1268	3.6	15
31	.1835	5.9	18	.2137	6.4	10	.7685	7.4	9	.4412	6.5	6
32	.2761	6	1	.2509	6.2	7	.8195	6.8	6	.6531	6.2	1
33	.2412	6	4	.2463	5.4	8	1.0415	5.4	3	.3128	4.6	8
34	.2334	4.8	5	.1939	5	13	.900	5.0	5	.1141	4.6	18
41	.1962	5.9	15	.2718	6.4	5	.6849	7.2	12	.5864	6.5	2
42	.2317	5.6	6	.2588	6.0	6	1.0149	7.0	4	.5081	6.8	4
43	.4988	5.0	14	.2752	5.4	3	1.2294	6.2	1	.1161	4.6	17
51	.2236	5.8	8	.2727	6.5	4	.7403	7.2	10	.4805	6.2	5
52	.2061	5.6	12	.3183	6.7	1	1.0735	7.2	2	.3031	6.2	9
61	.1399	5.2	21	.2885	6.8	2	.8054	8.4	7	.2531	6.2	10
Richards	.1995	4.8					.4515	6.8		.0952	5.6	
Pfeffer							.0973	4.6		.0497	3.8	
Currie							.0144	3.2				
Meyer							.4187	5.2		.1473	3.8	
Czapek							.1053	7.4		.3126	6.8	

* Average of three cultures.

Figures 1, 2, 3, and 4 represent the arrangement of the triangular system. The area within the dotted line in each case represents the seven cultures making the best growth.

The results of this experiment show clearly that a proper balance of the inorganic constituents in the solution is very essential, and that this balance can be readily obtained by the use of the triangular system. Using approximately the same concentration as Richards' solution and a constant source of carbon, the growth obtained in the balanced solutions was much superior for every organism used in the experiment.

SUMMARY AND CONCLUSIONS

1. Various synthetic solutions have been evolved to meet the needs of specific organisms. Fungi vary so much in their mineral and food re-

quirements that no one medium can be made to give satisfactory results with all organisms.

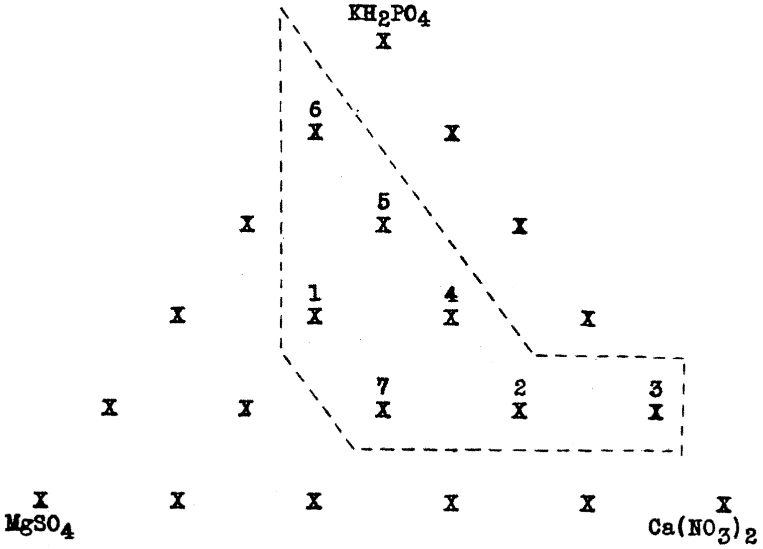

Fig. 1.
Fusarium oxysporum.

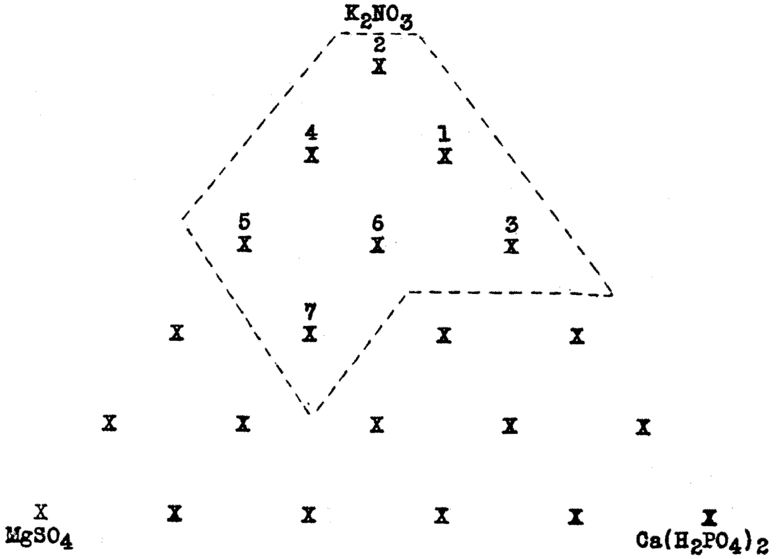

Fig. 2.
Fusarium oxysporum.

2. Zinc has a stimulating effect in the case of a limited number of organisms. In the case of some 15 species of parasitic organisms, zinc did not accelerate growth.

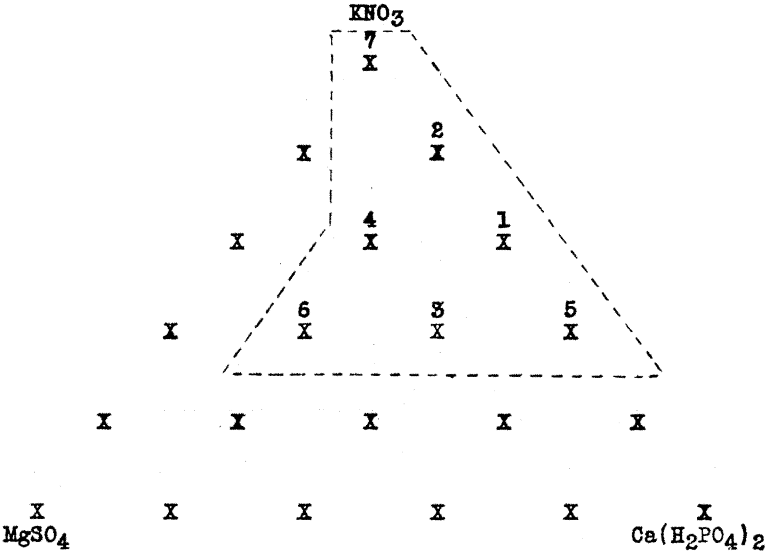

Fig. 3.
Macrosporium sarcinaeforme.

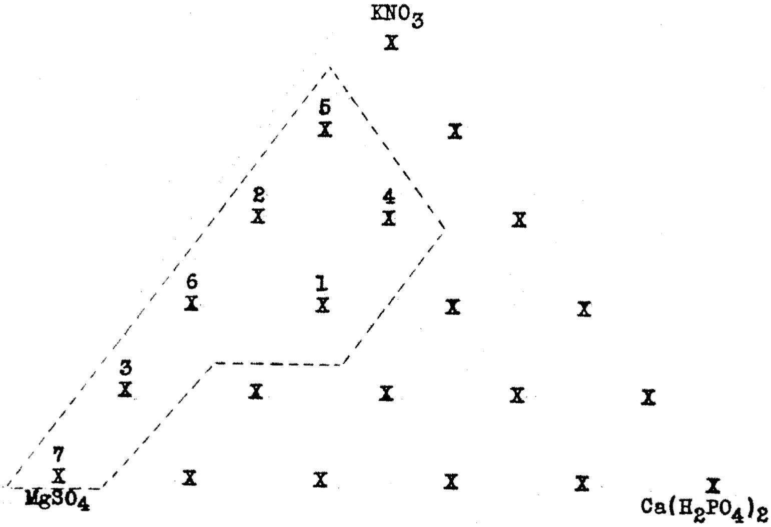

Fig. 4.
Phoma apiicola.

3. Calcium, in the case of the organisms used, proved to be beneficial in the majority of instances and seems to have a place, probably in the correcting of acidity, in the best synthetic solutions.

4. The inorganic elements in a synthetic solution should be properly balanced. The balancing of these salts is a comparatively simple procedure when the three-salt or triangular system is used. By this system the mineral requirements of any particular fungus can be quickly and accurately ascertained. By then selecting the sugar most readily used, a favorable culture solution can be made.

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